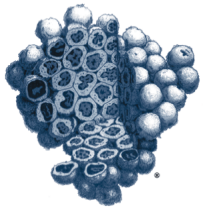

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Identification of Long-Term Repopulating Potential of Human Cord Blood-Derived CD34 –flt3– Severe Combined Immunodeficiency-Repopulating Cells by Intra-Bone Marrow Injection

Takafumi Kimura, Rumiko Asada, Jianfeng Wang, Takashi Kimura, Miho Morioka, Kazuo Matsui, Katsuya Kobayashi, Kae Henmi, Shiro Imai, Masakazu Kita, Takashi Tsuji, Yutaka Sasaki, Susumu Ikehara and Yoshiaki Sonoda

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Identification of Long-Term Repopulating Potential of Human Cord Blood-Derived CD34⁻flt3⁻ Severe Combined Immunodeficiency-Repopulating Cells by Intra-Bone Marrow Injection

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Key Words. Flt3 • Severe combined immunodeficiency-repopulating cell • Intra-bone marrow injection • Cord blood • Hematopoiesis

ABSTRACT

Recently, we have identified human cord blood (CB)-derived CD34-negative (CD34⁻) severe combined immunodeficiency (SCID)-repopulating cells (SRCs) using the intra-bone marrow injection (IBMI) method (Blood 2003; 101:2924). In contrast to murine CD34⁻ Kit⁺Sca-1⁺Lineage⁻ (KSL) cells, human CB-derived Lin⁻CD34⁻ cells did not express detectable levels of c-kit by flow cytometry. In this study, we have investigated the function of flt3 in our identified human CB-derived CD34⁻ SRCs. Both CD34⁺flt3$^{+/-}$ cells showed SRC activity. In the CD34⁻ cell fraction, only CD34⁻flt3⁻ cells showed distinct SRC activity by IBMI. Although CD34⁺flt3⁺ cells showed a rather weak secondary repopulating activity, CD34⁺flt3⁻ cells repopulated many more secondary recipient mice. However, CD34⁻flt3⁻ cells repopulated all of the secondary recipients, and the repopulating rate was much higher. Next, we cocultured CD34⁻flt3⁻ cells with the murine stromal cell line HESS-5. After 1 week, significant numbers of CD34⁺flt3$^{+/-}$ cells were generated, and they showed distinct SRC activity. These results indicated that CB-derived CD34⁻flt3⁻ cells produced CD34⁺flt3⁻ as well as CD34⁺flt3⁺ SRCs in vitro. The present study has demonstrated for the first time that CB-derived CD34⁻ SRCs, like murine CD34⁻ KSL cells, do not express flt3. On the basis of these data, we propose that the immunophenotype of very primitive long-term repopulating human hematopoietic stem cells is Lin⁻CD34⁻c-kit⁻flt3⁻. STEM CELLS *2007;25:1348–1355*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

It is well documented that the tyrosine kinase receptors c-kit and flt3 are expressed and function in early mouse [1–10] and human hematopoiesis [1, 11–21]. Moreover, their respective ligands, stem cell factor (SCF) and flt3 ligand (FL), synergistically act with each other and play an important role in the regulation (generation, maintenance, proliferation, differentiation, and expansion) of early stages of murine and human candidate hematopoietic stem cells (HSCs) [1–21]. The most primitive HSCs in mammals, including mice and humans, have long been believed to be CD34 antigen-positive (CD34⁺) [22]. However, Osawa et al. [23] revealed that murine long-term lymphohematopoietic reconstituting HSCs are lineage marker-negative (Lin⁻) c-kit⁺Sca-1⁺CD34-low/negative (CD34$^{lo/-}$ KSL). In a murine model, it was recently reported that flt3⁻ KSL cells supported long-term multilineage hematopoietic reconstitution [24]. In contrast, flt3⁺ KSL cells are progenitors for the common lymphoid stage [24]. These flt3⁺ KSL cells have also been shown to lack erythro-megakaryocytic potential [25]. This notion was supported by the other reports that mice deficient in the expression of flt3 or FL showed deficient lymphopoiesis [26–28].

Recently, using the intra-bone marrow injection (IBMI) method, we have successfully identified human cord blood (CB)-derived CD34-negative (CD34⁻) severe combined immunodeficiency (SCID)-repopulating cells (SRCs) with extensive lymphoid and myeloid repopulating ability [29]. These CD34⁻ SRCs seemed to be more primitive HSCs than CD34⁺ SRCs [29, 30]. They could home into the BM niche only by IBMI, because they expressed lower levels of homing receptors, including CXCR4, and had poor SDF-1/CXCR4-mediated migration ability [29]. In contrast to the murine candidate HSCs

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(CD34$^-$ KSL cells) [23], our identified CD34$^-$ SRCs did not express detectable levels of c-kit tyrosine kinase receptor by flow cytometry. However, the degree to which flt3 is expressed on human HSCs, including CD34$^+$ and CD34$^-$ SRCs, which are capable of in vivo lymphomyeloid reconstitution, has not been fully elucidated.

Until now, a number of studies have reported that flt3 is expressed and functioned in the human CD34$^+$ hematopoietic progenitor cells [11–17, 19, 20], including long-term culture-initiating cells (LTC-ICs) [15, 20, 31]. However, only two reports have demonstrated, using the conventional intravenous injection method, that human CB- and bone marrow (BM)-derived CD34$^+$ HSCs capable of multilineage reconstitution in nonobese diabetic (NOD)/SCID mice express flt3 tyrosine kinase receptor [31, 32].

In this study, we have investigated, using the IBMI method, the function of flt3, which is expressed in early mouse [1–10] and human [11–21] hematopoiesis like c-kit, in our identified very primitive human CB-derived CD34$^-$ SRCs [29, 30], as well as more committed CD34$^+$ SRCs. Our data clearly demonstrate that part of human CB-derived CD34$^+$ SRCs express flt3, as reported previously [31, 32]. However, only CD34$^+$flt3$^-$ cells showed significant secondary repopulating ability, even when a comparable number of CD34$^+$flt3$^-$ cells as CD34$^+$flt3$^+$ cells was transplanted. Moreover, CD34$^-$flt3$^-$ cells showed distinct and potent SRC activity by IBMI, and they showed high and efficient secondary repopulating ability compared with CD34$^+$flt3$^-$ cells. The CD34$^-$flt3$^-$ cells also produced CD34$^+$flt3$^-$ and CD34$^+$flt3$^+$ SRCs after the coculture with the murine stromal cell line HESS-5 in vitro, suggesting that these CD34$^-$flt3$^-$ cells contained very primitive human CB-derived HSCs. The results of the present study are consistent with recent studies of the significance of flt3 expression in murine primitive hematopoiesis [24–28] and provide a new concept of hierarchy in the human primitive HSC compartment.

Materials and Methods

Collection of CB Samples and Processing

CB samples were obtained from normal full-term deliveries with signed informed consent and approved by the institutional review boards of Kansai Medical University and Kyoto Prefectural University of Medicine. CB-derived mononuclear cells (MNCs) were isolated using Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden; http://www.amersham.com) density gradient centrifugation. The MNCs were further enriched by negative depletion of eight lineage-positive cells, including CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and Glycophorin A using a StemSep device (StemCell Technologies, Vancouver, BC, Canada; http://www.stemcell.com), as reported previously[29, 30].

Purification of Lin$^-$CD34$^+$flt3$^{+/-}$ and Lin$^-$CD34$^-$flt3$^{+/-}$ Cells

The above-mentioned lineage-negative (Lin$^-$) cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 monoclonal antibody (mAb) (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), PC5-conjugated anti-CD34 mAb (Beckman Coulter), and biotinylated anti-flt3 mAb (M22, Immunex, Seattle, http://immunex.com) followed by incubation with streptavidin-phycoerythrin (SA-PE; Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com), as reported previously [20, 29, 30]. These stained cells were then sorted into four fractions, including CD34$^+$flt3$^{+/-}$ and CD34$^-$flt3$^{+/-}$ cells, as shown in Figure 1C using a FACSVantage (Becton Dickinson) as reported [29, 30]. The viability of these sorted cells was consistently more than 99%. Approximately 80% of the CD34$^+$ cell fraction in these Lin$^-$ cells expressed flt3 receptor. On the other hand, only 30% of the CD34$^-$ cell fraction in the same Lin$^-$ cells expressed this receptor. In separate experiments, we stained these immunomagnetically separated Lin$^-$ cells with 13 FITC-conjugated lineage-specific mAbs as reported previously [29], PC5-conjugated anti-CD34 mAb (Beckman Coulter), and PE-conjugated anti-c-kit mAb (Beckman Coulter) and examined the expression pattern of c-kit receptor (Fig. 1D).

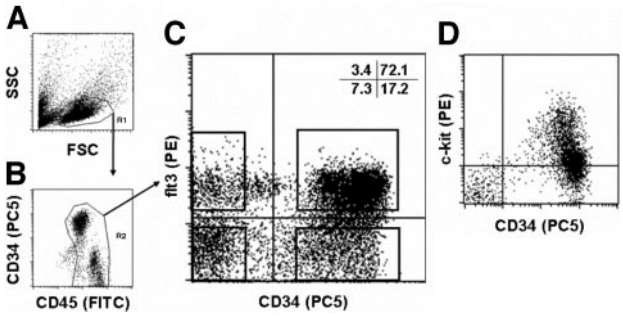

Figure 1. Expression of flt3 or c-kit receptor on cord blood-derived Lin$^-$ cells. **(A):** The forward scatter/SSC profile of immunomagnetically separated Lin$^-$ cells. The R1 gate was set on the lymphocyte window. **(B):** Lin$^-$CD45$^+$CD34$^{+/-}$ cells present in R1 gate were gated as R2. **(C):** The expression pattern of CD34 and flt3 on R2 gated cells is shown. Cells residing in the four cell fractions were classified as Lin$^-$CD34$^+$flt3$^+$, Lin$^-$CD34$^+$flt3$^-$, Lin$^-$CD34$^-$flt3$^+$, and Lin$^-$CD34$^-$flt3$^-$ cells, respectively. Each sorting window is shown as a solid square. Figures in upper right corner show percentages of cells in each quadrant. **(D):** The expression pattern of c-kit on Lin$^-$CD34$^{+/-}$ cells in a separate experiment. Abbreviations: FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

Clonal Cell Culture

Human colony-forming cells (CFCs) were assayed using our standard methylcellulose cultures as reported previously [18–21, 29, 30, 33, 34]. Briefly, 200 or 500 sorted Lin$^-$CD34$^{+/-}$flt3$^{+/-}$ cells were plated in 1 ml of culture containing 1.2% methylcellulose (Shinetsu Chemical, Tokyo, http://www.shinetsu.co.jp/e), 30% fetal calf serum (FCS; Hyclone, Laboratories, Logan, UT, http://www.hyclone.com), 1% bovine serum albumin (Sigma-Aldrich, St Louis, http://www.sigmaaldrich.com), 5×10^{-5} mol/L 2-mercaptoethanol (Sigma-Aldrich), and various recombinant human (rh) cytokines, including SCF, interleukin (IL)-3, granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and erythropoietin (Epo) in 35-mm Lux suspension culture dishes (Nunc, Rochester, NY, http://www.nuncbrand.com). For culture of megakaryocyte colony-forming cell (CFU-Meg), 10% platelet-poor plasma (PPP) [33] instead of 30% FCS, and rh thrombopoietin (TPO) were used. Cytokines, including G-CSF, Epo, and TPO were provided by Kirin Brewery Company (Takasaki, Japan, http://www.kirin.com). SCF, IL-3, and GM-CSF were purchased from R&D Systems Inc. (Minneapolis, http://www.rndsystems.com). Dishes were incubated at 37°C in a fully-humidified atmosphere flushed with a combination of 5% CO_2, 5% O_2, and 90% N_2. On days 12–14 of incubation, all colonies were scored under an inverted microscope according to their typical morphologic features, as reported elsewhere [18–21, 29, 30, 33]. CFU-Meg-derived pure megakaryocyte colonies were identified in situ as clusters of large cells, which were highly refractile and showed irregular contour and hyaline nongranulated cytoplasm. The types of colonies identified in situ were granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM), erythroid burst (BFU-E), erythrocyte-containing mixed (CFU-Mix), and the above-mentioned CFU-Meg. The numbers of all types of hematopoietic colonies were determined as the mean of three independent experiments.

IBMI of Purified Cells

IBMI was carried out as reported previously [29, 30, 35]. Briefly, after sterilization of the skin around the left knee joint, the knee was flexed to 90 degrees and the proximal side of the tibia was drawn to the anterior. A 27-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and then inserted into the BM cavity. Using a Hamilton's microsyringe, the number-specified donor cells per under 10 μl of α-medium were carefully and slowly injected from the bone hole into the BM cavity.

SCID-Repopulating Cell Assay

An SRC assay was performed using the methods reported previously [36, 37] with modifications [29, 30, 38]. Five-week-old NOD/Shi-*scid*/*scid* (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan, http://www.clea-japan.com). The animal experiments were approved by the Animal Care Committees of Kansai Medical University and Kyoto Prefectural University of Medicine. All mice were handled in sterile conditions and maintained in germ-free isolators located in the Central Laboratory Animal Facilities of Kansai Medical University and Kyoto Prefectural University of Medicine. In this study, purified 3×10^4 to 5×10^4 CB-derived Lin$^-$CD34$^+$flt3$^{+/-}$, or 2×10^4 to 7×10^4 CB-derived Lin$^-$CD34$^-$flt3$^{+/-}$ cells were transplanted by IBMI into sublethally irradiated (250 cGy using a ^{137}Cs-γ irradiator) 8–12-week-old mice. As we reported previously [29, 30], CB-derived CD34$^-$ SRCs were detected only by the IBMI technique. Moreover, the repopulation rate of CD34$^+$ SRCs by IBMI was significantly higher than that by the conventional tail-vein injection method [29]. Therefore, we used the IBMI technique to analyze SRC activities of Lin$^-$CD34$^{+/-}$Flt3$^{+/-}$ cells in this study. The mice were killed 8–12 weeks after transplantation, and the BMs from the pairs of femurs, tibiae, and humeri of each mouse were flushed into α-medium. The rates of human CD45$^+$ cells in the murine BMs were analyzed by flow cytometry (FACS Calibur; Becton Dickinson) as described in the next section. Mice were scored as positive if over 0.1% of total murine BM cells were human CD45$^+$.

Analysis of Human Cell Engraftment in NOD/SCID Mice by Flow Cytometry

The repopulation of human hematopoietic cells in murine BMs was determined by detecting the number of cells positively stained with PC5-conjugated anti-human CD45 mAb (Beckman Coulter) by flow cytometry. The cells were also stained with PE-conjugated anti-human CD34 mAb (Becton Dickinson), and FITC-conjugated mAbs for human lineage-specific Ags, including CD19 (eBioscience, San Diego, http://www.ebioscience.com), and CD33 (Beckman Coulter) for the detection of human lymphoid and myeloid hematopoietic cells, respectively.

Secondary Transplantation

For secondary transplantations, murine BM cells were obtained from the pairs of femurs, tibiae, and humeri of moderately engrafted primary recipient mice 8–12 weeks after transplantation with 3×10^3 to 5×10^3 Lin$^-$CD34$^+$flt3$^+$, 4×10^3 to 5×10^3 Lin$^-$CD34$^+$flt3$^-$, or 2×10^4 to 3×10^4 Lin$^-$CD34$^-$flt3$^-$ cells, respectively. The human cell repopulation rates in the primary recipients' BMs were comparable and approximately 4%–8%. Whole BM cells were transplanted by IBMI into sublethally (250 cGy) irradiated secondary recipient mice. Eight to 10 weeks after transplantation, the presence of human CD45$^+$ cells in the secondary recipients' BMs was analyzed by flow cytometry, as described for primary transplantation.

Coculture with HESS-5 Cells and SRC Activity of Culture-Generated CD34$^+$flt3$^{+/-}$ Cells

A total of 5×10^4 purified Lin$^-$CD34$^-$flt3$^-$ cells per 12.5-cm^2 culture flask (BD Falcon; Becton Dickinson) onto preestablished irradiated HESS-5 [39] layers in StemPro-34 medium (Gibco Laboratories, Grand Island, NY, http://www.invitrogen.com) and a cocktail of cytokines, including 300 ng/ml SCF (R&D), 300 ng/ml TPO (Kirin), 10 ng/ml IL-3 (R&D), 10 units/ml IL-6 (provided by Dr. Akira Okano, Ajinomoto Co. Inc., Yokohama, Japan, http://www.ajinomoto.com), 10 ng/ml G-CSF (Kirin), and 5% FCS (Hyclone). After 1 week, all cells were collected by vigorous pipetting, and stained with PC5-conjugated anti-CD34 mAb (Beckman Coulter) and biotinylated anti-flt3 mAb (Immunex) as mentioned herein. Cells were then stained with SA-PE (Becton Dickinson). The rates of CD34$^+$flt3$^{+/-}$ cells were analyzed by flow cytometry. Simultaneously, these CD34$^+$flt3$^{+/-}$ cells were separately obtained by cell sorting (FACSVantage) for the detection of SRC activity. One to 2×10^4 CD34$^+$flt3$^+$ or 2×10^4 to 4×10^4 CD34$^+$flt3$^-$ cells were transplanted by IBMI into sublethally (250 cGy) irradiated recipient mice. Eight weeks after transplantation, the presence of human CD45$^+$ cells in the recipients' BMs was analyzed by flow cytometry, as described for primary transplantation.

Statistical Analysis

The significance of differences in the SRC assays and the numbers of hematopoietic colonies was determined using the Mann-Whitney *U* test and the two-tailed Student's *t* test, respectively.

RESULTS

Expression of flt3 and c-kit Receptors on Lin$^-$CD45$^+$CD34$^{+/-}$ Cells

First, we depleted the eight lineage-positive cells from CB-derived MNCs using the immunomagnetic beads system [29, 30]. Then, Lin$^-$CD45$^+$CD34$^{+/-}$ cells were gated as R2 as shown in Figure 1B. These Lin$^-$CD45$^+$ cells were subdivided into four distinct populations on the basis of their surface CD34 and flt3 expression (Fig. 1C). We sorted these four fractions for further stem cell characterization. The phenotypic purity of the sorted cells consistently exceeded 98% when checked using postsorting flow cytometric analysis (data not shown). Importantly, these Lin$^-$CD34$^-$ cells did not express detectable levels of c-kit receptors by flow cytometry, as shown in Figure 1D.

Characteristics of Colony-Forming Capacity by CB-Derived Lin$^-$CD34$^{+/-}$flt3$^{+/-}$ Cells

The colony-forming capacities of these four fractions were quite different. The plating efficiency of each Lin$^-$CD34$^+$flt3$^+$ or Lin$^-$CD34$^+$flt3$^-$ cell fraction was approximately 50% and comparable (Fig. 2A). Lin$^-$CD34$^+$flt3$^+$ cells contained approximately 81% CFU-GM, 17% BFU-E, and 2% CFU-Mix. In contrast, Lin$^-$CD34$^+$flt3$^-$ cell fraction contained 21% CFU-GM, 66% BFU-E, and 12% CFU-Mix. The Lin$^-$CD34$^-$flt3$^{+/-}$ cell fractions showed almost no colony formation (data not shown). On the other hand, the vast majority of CFU-Megs (more than 90%) were detected in the Lin$^-$CD34$^+$flt3$^-$ cell fraction (Fig. 2B).

These results clearly demonstrate that CB-derived Lin$^-$CD34$^+$flt3$^+$ cells display weak erythroid and megakaryocytic potentials. These findings were consistent with a recent study in which murine flt3$^+$ KSL cells failed to produce significant erythroid and megakaryocytic progeny [25].

SRC Activity and Lymphomyeloid-Reconstituting Capacity of CB-Derived Lin$^-$CD34$^{+/-}$flt3$^{+/-}$ Cells by IBMI

In this study, we have investigated the function of flt3 in our identified human CB-derived CD34$^-$ SRCs. First, we studied the SRC activity of CB-derived Lin$^-$CD34$^+$flt3$^{+/-}$ or CD34$^-$flt3$^{+/-}$ cells using IBMI, as shown in Figure 3. Both CD34$^+$flt3$^{+/-}$ cells repopulated all 20 recipient mice (10 mice each). The level of human CD45$^+$ cells in the murine BMs that

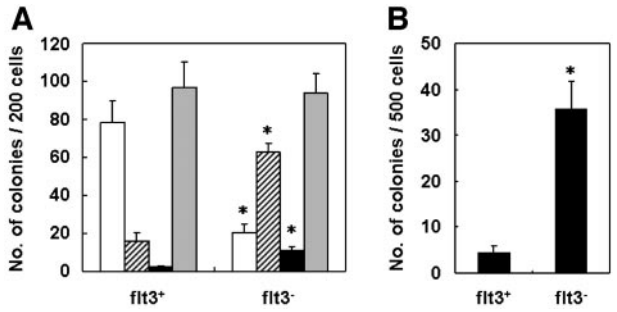

Figure 2. Colony-forming capacities of $Lin^-CD34^+flt3^{+/-}$ cells. **(A):** The colony-forming capacities of 200 $Lin^-CD34^+flt3^{+/-}$ cells in the presence of stem cell factor, interleukin-3, granulocyte macrophage (GM) colony-stimulating factor (CSF), granulocyte (G) CSF, and erythropoietin. Open, shaded, closed, and gray bars represent the number of granulocyte/macrophage colony-forming units (CFUs; including CFU-G, CFU-macrophage, and CFU-GM), erythroid burst, CFU-Mix, and total colony, respectively. **(B):** The colony-forming capacities of 500 $Lin^-CD34^+flt3^{+/-}$ cells in the presence of thrombopoietin. Closed bars represent the number of megakaryocyte CFUs. The numbers of all types of colonies were determined as the mean of three independent experiments. Vertical bars represent standard deviation, and asterisks show statistical significance ($p < .01$) between the numbers of designated colonies formed by $flt3^+$ and $flt3^-$ cells, respectively.

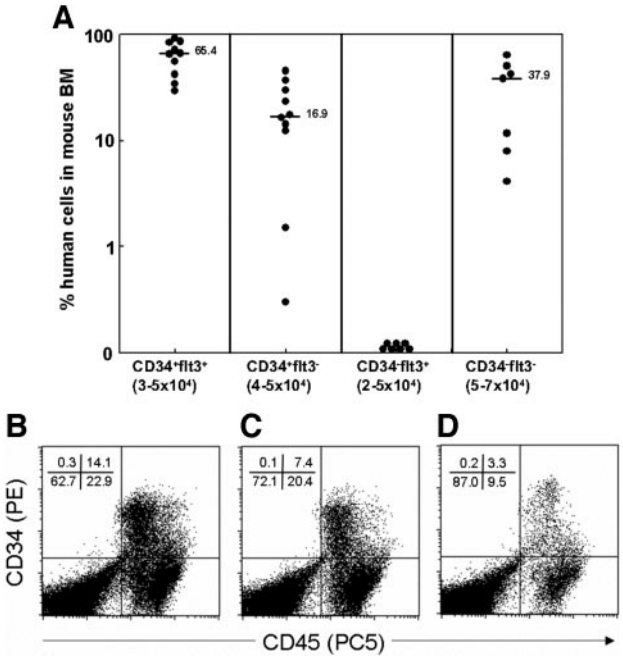

Figure 3. Severe combined immunodeficiency-repopulating cell activities of $Lin^-CD34^{+/-}flt3^{+/-}$ cells by intra-BM injection (IBMI). **(A):** Each mouse transplanted with designated numbers of cord blood-derived $Lin^-CD34^+flt3^+$, $Lin^-CD34^+flt3^-$, $Lin^-CD34^-flt3^+$, and $Lin^-CD34^-flt3^-$ cells was sacrificed 8–12 weeks after transplantation. Closed circles represent the repopulation rates in total murine BMs by the IBMI, respectively. Horizontal bars represent each median of the repopulation rates. **(B–D):** The human $CD45^+$ cell reconstitution in the representative mouse presented in **(A)** received transplants of $CD34^+flt3^+$ **(B)**, $CD34^+flt3^-$ **(C)**, and $CD34^-flt3^-$ **(D)** cells, respectively. Percentages of cells in each quadrant are presented in the upper left corner. Abbreviations: BM, bone marrow; PE, phycoerythrin.

received transplants of $CD34^+flt3^+$ cells ($n = 10$; 29.3% to 90.8%; median, 65.4%) was higher than those that received transplants of $CD34^+flt3^-$ cells ($n = 10$; 0.3% to 45.1%; median, 16.9%).

Table 1. Lymphomyeloid reconstitution abilities of $CD34^+flt3^{+/-}$ and $CD34^-flt3^-$ severe combined immunodeficiency repopulating cells by intra-bone marrow (BM) injection

	%	
Cells	**CD33**	**CD19**
$CD34^+flt3^+$	5.9	44.4
	4.5	**41.5**
	2.8	29.5
$CD34^+flt3^-$	1.3	16.9
	1.1	**6.1**
	0.5	2.1
$CD34^-flt3^-$	7.4	**19.3**
	3.3	21.1
	1.9	13.4

Each mouse transplanted with designated numbers of cord blood-derived $Lin^-CD34^+flt3^+$, $Lin^-CD34^+flt3^-$, and $Lin^-CD34^-flt3^-$ cells was sacrificed 8 to 12 weeks after transplantation. First, the R1 gate was set on the total murine BM cells obtained from these representative mice, and then human $CD45^+$ cells were gated as R2. Expression of lineage markers, including CD19 (lymphoid) and CD33 (myeloid) on the R2 gated cells, was analyzed by three color flow cytometry. Boldfacing represents each median of the repopulation rates.

On the other hand, the seven mice that received transplants of $CD34^-flt3^+$ cells did not show human cell repopulation. Only $CD34^-flt3^-$ cells repopulated all seven recipient mice, and the level of human $CD45^+$ cells in the murine BMs was 4.1% to 63.3% (median, 37.9%). These results indicated for the first time that the CB-derived $Lin^-CD34^-flt3^-$ cell population contained SRCs, as detected by IBMI.

To further evaluate the function of flt3 expression in $CD34^+$ and $CD34^-$ SRCs, we studied their lymphomyeloid reconstitution abilities using IBMI. In our SRC assay system, all NOD/SCID mice transplanted either with 3×10^4 to 5×10^4 $Lin^-CD34^+flt3^{+/-}$ cells or 5×10^4 to 7×10^4 $Lin^-CD34^-flt3^-$ cells by IBMI showed signs of human cell engraftment. The analyses of the three representative mice transplanted either with $Lin^-CD34^+flt3^{+/-}$ cells or $Lin^-CD34^-flt3^-$ cells clearly indicate that these three classes of SRCs have an extensive differentiation capacity to B-lymphoid (CD19) and myeloid (CD33) lineages in vivo (Table 1).

Next, the percentages of lineage-positive cells expressing CD19 and CD33 were compared (Table 1). These results demonstrated that all three classes of SRCs could supply lymphoid as well as myeloid cells at 8–12 weeks after the transplantation. Interestingly, $CD34^+flt3^+$ SRCs showed a lymphoid-dominant repopulation pattern compared with the other two classes of SRCs. These results are consistent with the notion that cells in the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ murine HSC compartment coexpressing flt3 tyrosine kinase receptor sustain lymphoid potential [24, 25], and also that mice deficient in the expression of flk2/flt3 or FL show deficient lymphopoiesis [26–28].

Secondary Repopulating Ability of $Lin^-CD34^+flt3^{+/-}$ or $Lin^-CD34^-flt3^-$ Cells by IBMI

To further evaluate the long-term repopulating potential of these three populations ($CD34^+flt3^+$, $CD34^+flt3^-$, and $CD34^-flt3^-$ cells), BM cells obtained from each engrafted primary recipient mouse were assessed for their SRC activity by secondary transplantation by IBMI. Only one of six mice that received whole BM cells obtained from primary recipient mice that received transplants of $CD34^+flt3^+$ cells showed secondary repopulating activity (Fig. 4). On the other hand, 83% (five of six) of the

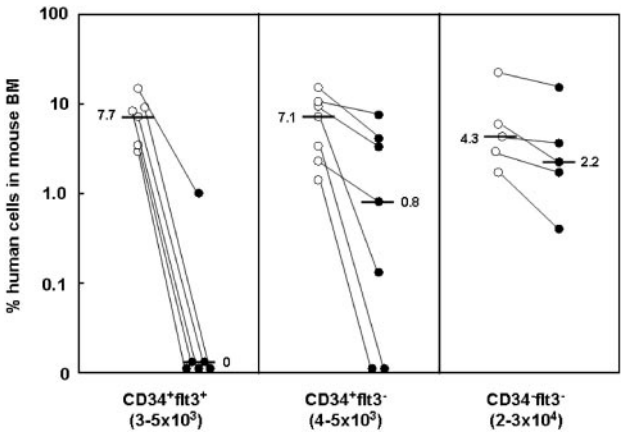

Figure 4. Secondary repopulating capacities of $Lin^-CD34^+flt3^{+/-}$ or $Lin^-CD34^-flt3^-$ cells. Cells transplanted to primary recipients (PRs) by intra-BM injection (IBMI) numbered 3×10^3 to 5×10^3 $CD34^+flt3^+$cells, 4×10^3 to 5×10^3 $CD34^+flt3^-$ cells, or 2×10^4 to 3×10^4 $CD34^-flt3^-$ cells. Human cell repopulations of BMs in PRs (open circles) analyzed 8–12 weeks after transplantation were comparable and 4%–8%. Whole BM cells obtained from PRs were transplanted to secondary recipients (SRs) by IBMI. Human cell repopulation in SRs (closed circles) was analyzed 8–10 weeks after secondary transplantation. Horizontal bars represent each median of the repopulation rates in PRs and SRs, respectively. Abbreviation: BM, bone marrow.

secondary recipients that received whole BM cells from primary recipients that received $CD34^+flt3^-$ cells could be repopulated. Moreover, all five secondary recipient mice that received whole BM cells from primary recipients that received $CD34^-flt3^-$ cells could be repopulated with a higher secondary repopulating rate (Fig. 4). These results demonstrated that $CD34^-flt3^-$ SRCs have more potent secondary reconstituting abilities in comparison with the other two types of SRCs, and could sustain long-term human hematopoiesis in NOD/SCID mice.

SRC Activity of Culture-Generated $CD34^+flt3^{+/-}$ Cells by IBMI

Recently, we reported that our identified $CD34^-$ SRCs could produce $CD34^+$ SRCs after being cocultured with the murine stromal cell line HESS-5 [29]. Therefore, we cocultured $CD34^-flt3^-$ cells with HESS-5 in the presence of SCF, TPO, IL-3, IL-6, and G-CSF, as reported previously [29]. After 1 week, significant numbers of $CD34^+flt3^-$ and $CD34^+flt3^+$ cells were generated, as shown in Figure 5C. We then sorted these two populations ($CD34^+flt3^{+/-}$ cells) and tested their SRC activities by IBMI. Seven of 10 and 5 of 10 mice that received either $CD34^+flt3^+$ or $CD34^+flt3^-$ cells were repopulated with human cells. (Table 2). Human cell repopulation rates in mice that received transplants of either $CD34^+flt3^+$ or $CD34^+flt3^-$ cells were 1.2%–8.8% (median, 4.5%) and 1.4%–7.8% (median, 3.7%), respectively.

DISCUSSION

A number of studies have demonstrated that flt3 tyrosine kinase receptor plays a pivotal role in the regulation of primitive murine [1–10] and human [11–17, 19, 20] hematopoietic stem/progenitor cells such as c-kit. In the murine model, the expression and functional significance of flt3 and c-kit receptors in early hematopoiesis has been investigated extensively [1–10, 23–28, 40–42]. Many studies have suggested that murine pluripotent long-term repopulating hematopoietic stem cells (LTR-HSCs) express c-kit [40–42]. Particularly, Osawa et al. [23] clearly indicated that a single $CD34^-$ KSL cell efficiently reconstituted as many as one of five recipient mice. However, they did not rule out the possibility of coexistence of a less frequent $c\text{-}kit^-$ LTR-HSC, which might fail to home into the BM niche by intravenous injection. In support of the potential existence of $c\text{-}kit^-$ murine HSCs, several studies have reported the existence of LTR-HSCs with little or no c-kit expression [43–45]. Among them, Doi et al. [43] clearly demonstrated that candidate HSC could be subdivided into $c\text{-}kit^{low}$ and $c\text{-}kit^{<low}$ (no detectable cell surface expression but positive for mRNA expression) populations. Both populations could support donor-type long-term multilineage reconstitution in primary recipients. However, only $c\text{-}kit^{<low}$ HSCs showed secondary and tertiary reconstituting capacity. In addition, Ortiz et al. [44] reported that $c\text{-}kit^-$ pluripotent stem cells can give rise to $c\text{-}kit^+$ cells with colony-forming unit in spleen (CFU-S) activity, suggesting that $c\text{-}kit^+$ HSCs are recruited from a more primitive quiescent $c\text{-}kit^-$ HSC population. Collectively, these reported studies suggest that most of the murine LTR-HSCs express a low to high level of c-kit on their surfaces, but also that there is a less frequent subpopulation expressing less than a low level of c-kit coexisting in murine BMs.

On the other hand, the flt3 receptor has also been shown to be expressed and to function in murine candidate HSCs [1–10, 24–28], including $Lin^-Sca\text{-}1^+AA4^+$ fetal liver cells [5], $CD34^{+/-}$ KSL BM cells [10, 24–26], and Thy-1.1^{lo}KLS cells [9]. In particular, Jacobsen et al. have extensively studied the expression and functional significance of flt3 receptor on mu-

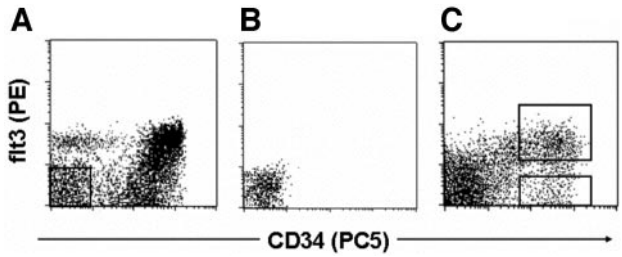

Figure 5. Expression pattern of CD34 and flt3 on sorted $Lin^-CD34^-flt3^-$ cells after the 7-day coculture with HESS-5 cells. **(A):** Flow cytometry pattern of immunomagnetically separated cord blood-derived Lin^- cells stained with anti-flt3 (PE) and anti-CD34 (PC5) monoclonal antibodies. $Lin^-CD34^-flt3^-$ cells were sorted for the coculture with HESS-5 cells. The sorting gate is indicated by the solid square. **(B):** Postsorting analysis of the sorted $Lin^-CD34^-flt3^-$ cells. **(C):** The expression pattern of flt3 on $CD34^+$ cells derived from the 7-day cocultures of sorted $Lin^-CD34^-flt3^-$ cells with the murine stromal cell line, HESS-5, in the presence of a cocktail of cytokines. The sorting gates for culture-generated $CD34^+flt3^{+/-}$ cells are indicated by two solid squares. Abbreviation: PE, phycoerythrin.

Table 2. Severe combined immunodeficiency (SCID) repopulating cell activity of culture-generated $CD34^+flt3^{+/-}$ cells

Sorted cell fraction	No. of cells transplanted	Engraftment	Human $CD45^+$/cells (%) Range	Median
$CD34^-fit3^+$ cells	1×10^4 to 2×10^4	7/10	1.2–8.8	4.5
$CD34^+flt3^-$ cells	2×10^4 to 4×10^4	5/10	1.4–7.8	3.7

Designated numbers of culture-generated $CD34^+fit3^+$ and $CD34^+flt3^-$ cells obtained by cell sorting were transplanted to nonobese diabetic/SCID mice by intra-bone marrow (BM) injection. After 8 weeks, repopulation of human $CD45^+$ cells in murine BMs was analyzed.

rine LTR-HSCs [7, 8, 10, 24–26]. Adolfsson et al. [24] first reported that the upregulation of flt3 on BM-derived KSL cells is accompanied by loss of self-renewal capacity. In other words, $flt3^+$ KSL cells rapidly and efficiently reconstituted B and T lymphopoiesis, and only $flt3^-$ KSL cells supported sustained multilineage reconstitution [24–26]. On the basis of these data, they proposed that $flt3^+$ KSL cells are progenitors for the common lymphoid progenitor (CLP) [24–26]. Earlier, Kondo et al. [46] identified other CLPs that have the $Lin^-Thy\text{-}1^-Sca\text{-}1^{low}c\text{-}kit^{low}IL\text{-}7R\alpha^+$ immunophenotype. In Adolfsson's study [25], it was shown that the $flt3^+$ KSL cells produced this $IL\text{-}7R\alpha^+$ CLP in vitro as well as in vivo. These results suggested that the $flt3^+$ KSL population is distinct and most likely an intermediate between $flt3^-$ KSL (LTR-HSC) and this $IL\text{-}7R\alpha^+$ CLP. Interestingly, $flt3^+$ KSL cells were found to be almost exclusively $CD34^+$, whereas $flt3^-$ KSL cells contained a small but significant (5%) fraction of $CD34^-$ cells [24]. This notion was further supported by the recent study reported by Sitnicka et al. [26]. In this study, FL-deficient mice had severely (10-fold) reduced levels of CLP, although the numbers of common myeloid progenitors (CMPs) and $CD34^-$ KSL were unaffected [26]. Very recently, Adolfsson et al. [25] have clearly demonstrated that the herein-mentioned $flt3^+$ KSL cells sustain granulocyte, monocyte, and B- and T-cell potential, but fail to produce significant erythroid and megakaryocytic progeny. On the basis of these observations, they proposed an alternative road map for adult mouse blood lineage commitment [25].

In contrast to murine LTR-HSC, the expression and functional significance of flt3 and c-kit on human LTR-HSC has yet to be fully elucidated. Earlier studies have shown that most, if not all, long-term culture-initiating cell (LTC-IC) are $c\text{-}kit^+$ [47, 48]. However, we observed that extended LTC-IC (ELTC-IC; assayed after 7–9 weeks of coculture with allogeneic BM stromal layer) are apparently enriched in a CB-derived $CD34^+c\text{-}kit^{low/-}$ cell population [21]. Of note was that ELTC-ICs assayed after 9 weeks of coculture were detected only in the $CD34^+c\text{-}kit^-$ cell population [21]. Our data are consistent with several other in vitro studies [49–51]. Sogo et al. [49] have clearly demonstrated that CB-derived $CD34^+c\text{-}kit^{<low}$ cells mature into $CD34^+c\text{-}kit^{low}$ and $CD34^+c\text{-}kit^+$ cells in vitro, suggesting that the upregulation of c-kit protein on $c\text{-}kit^{<low}$ cells is the first maturational step of human HSCs [49]. Enrichment of human BM-derived primitive HSCs in the $CD34^+c\text{-}kit^{low}$ fraction was also confirmed using long-term engraftment studies in preimmune fetal sheep [51]. The findings showed that BM-derived $CD34^+c\text{-}kit^{low}$ cells transplanted to fetal sheep sustained long-term donor-derived hematopoiesis (up to 16 months) [52]. There is, however, no direct evidence yet for a distinct population of $c\text{-}kit^-$ or $c\text{-}kit^{<low}$ human primitive HSCs with long-term repopulating potential. Such a stem cell population is likely to be present at very low frequency in human BM- or CB-derived hematopoietic cells. Recently, we identified very primitive $CD34^-$ SRCs in human CB detected only by the IBMI method [29, 30]. As shown in Figure 1D, the CB-derived Lin^-CD34^- cell population did not express detectable levels of c-kit protein by flow cytometry. Therefore, our identified $CD34^-$ SRCs may correspond to such $c\text{-}kit^-$ or $c\text{-}kit^{<low}$ LTR-HSCs [29, 30].

In contrast to c-kit, the information regarding flt3 expression on human LTR-HSCs is much more limited. Recently, Sitnicka et al. [31], using the conventional intravenous injection method, clearly demonstrated that human BM- or CB-derived $CD34^+$ HSC capable of multilineage engrafting NOD/SCID mice do express flt3 receptors. Moreover, they also showed that CB-derived $CD34^+flt3^-$ cells could repopulate recipient mouse BMs. On the basis of these data, they proposed that most BM- and CB-derived $CD34^+$ SRCs express flt3, and that the expression pattern of flt3 and c-kit receptors on primitive mouse and human HSCs is different and contrasting. However, they did not investigate the secondary repopulating capacity of $CD34^+flt3^{+/-}$ cells as well as the repopulation capacity of the $CD34^-$ counterpart.

In this study, we have investigated the SRC activity of CB-derived $Lin^-CD34^+flt3^{+/-}$ cells as well as $Lin^-CD34^-flt3^{+/-}$ cells using the IBMI method. First, we confirmed that CB-derived $Lin^-CD34^+flt3^{+/-}$ cells showed distinct SRC activity by IBMI (Fig. 3; Table 1). Interestingly, we demonstrated for the first time that $Lin^-CD34^-flt3^-$ cells showed significant and potent SRC activity by IBMI. Moreover, our secondary transplantation study clearly indicated that the secondary repopulating capacity is most potently observed in $CD34^-flt3^-$ cells in comparison with $CD34^+flt3^{+/-}$ cells (Fig. 4). Finally, we observed that these $Lin^-CD34^-flt3^-$ cells could produce $CD34^+flt3^{+/-}$ SRCs after being cocultured with HESS-5 cells in the presence of a cocktail of cytokines (Fig. 5). These results suggest that $CD34^-flt3^-$ SRCs are the precursor for $CD34^+flt3^{+/-}$ SRCs. On the basis of the results of our present study, we propose that the immunophenotype of very primitive human LTR-HSCs is $Lin^-CD34^-c\text{-}kit^-flt3^-$. Primitive human LTR-HSCs may express lower levels of c-kit and flt3 receptors on their surfaces when they commit to more mature short-term repopulating HSCs (STR-HSCs). It is still unclear whether such a distinct pattern of c-kit and flt3 expression might identify distinct subpopulations of LTR-HSC or STR-HSC within the human HSC hierarchy.

From another point of view, we and many other investigators have planned to expand candidate human HSCs ex vivo using several cytokines, including SCF, FL, TPO, and IL-6/soluble IL-6 receptor (or fusion protein) [53–56]. However, the present study and other reported studies [24–26, 41–43,49–52] have demonstrated/suggested that very primitive LTR-HSCs do not express their receptors, such as c-kit and flt3. Furthermore, $Lin^-CD34^-c\text{-}kit^-flt3^-$ cells are still heterogeneous, and putative human LTR-HSC may express other potentially important stem cell molecules. Therefore, for clinical application in the near future, further studies will be required to elucidate the proposed model of the human HSC hierarchy as well as to identify hitherto unidentified molecules that are important (indispensable) for stem cell expansion.

In conclusion, the present study provides evidence that human CB-derived $CD34^-$ SRCs do not express flt3 tyrosine kinase receptors, like murine candidate HSCs $CD34^-$ KSL cells. According to our data, the immunophenotype of human LTR-HSC is $Lin^-CD34^-c\text{-}kit^-flt3^-$. Therefore, further studies will be required to identify positive markers, such as Sca-1 for murine $CD34^-$ KSL cells, for these primitive human LTR-HSCs in the near future.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Identification of Long-Term Repopulating Potential of Human Cord Blood-Derived CD34 –flt3– Severe Combined Immunodeficiency-Repopulating Cells by Intra-Bone Marrow Injection

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